Visual Evidence of Acidic Environment Within Degrading Poly(lactic-co-glycolic acid) (PLGA) Microspheres

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Purpose. In the past decade, biodegradable polymers have become the materials of choice for a variety of biomaterials applications. In particular, poly(lactic-*co*-glycolic acid) (PLGA) microspheres have been extensively studied for controlled-release drug delivery. However, degradation of the polymer generates acidic monomers, and acidification of the inner polymer environment is a central issue in the development of these devices for drug delivery.

Methods. To quantitatively determine the intrapolymer acidity, we entrapped pH-sensitive fluorescent dyes (conjugated to 10,000 Da dextrans) within the microspheres and imaged them with confocal fluorescence microscopy. The technique allows visualization of the spatial and temporal distribution of pH within the degrading microspheres (1).

Results. Our experiments show the formation of a very acidic environment within the particles with the minimum pH as low as 1.5.

Conclusions. The images show a pH gradient, with the most acidic environment at the center of the spheres and higher pH near the edges, which is characteristic of diffusion-controlled release of the acidic degradation products.

KEY WORDS: PLGA microspheres; pH; confocal fluorescence microscopy; protein drug delivery.

INTRODUCTION

Biodegradable polymer devices show great potential for a variety of therapeutic applications including controlled release of proteins and nucleic acids and polymer scaffolds for tissue engineering or cell-based therapies (2–5). A serious obstacle, however, is the insufficient stability of the protein, nucleic acid, or cell when introduced into the environment of the polymer. Understanding the causes of this instability is crucial to successful development of these technologies and requires knowledge of the physico-chemical environment to which the entrapped therapeutic is exposed. For example, polymer degradation may result in a build-up of acidic by-products since many of the biodegradable polymers (polyesters, polyanhydrides, etc.) are made up of acidic monomers (6–9). As low-pH environments are known to be deleterious to some proteins and nucleic acids (10–12), the pH changes within these biodegradable devices are expected to be important. There has been evidence showing the influence of basic salts on the stability and release of proteins from delivery devices (13,14). Thus, quantifying the pH environment within the microspheres is critical for furthering our understanding of these delivery systems.

Several studies, using indirect methods, have shown the presence of an acidic environment within degrading polymer devices. For example, the degradation of PLGA tablets was shown to be faster on the inside of the tablet relative to the outside (8). Since the polymer degrades by hydrolysis and is acid-catalyzed, the conclusion was that a low-pH environment within the tablet caused the increased degradation rate. Others have used pH-sensitive dyes and confocal microscopy to measure the pH immediately surrounding degrading polyanyhydride tablets and have found it to be lower than that of the surrounding bulk media (15). Again, this was an indication of an acidic intrapolymer environment. Uchida *et al.* analyzed insulin released from PLGA microspheres and found it to be characteristic of the protein which had been exposed to an acidic environment (16). In addition, Shenderova *et al.* developed several methods to examine the pH within microspheres and films, and the conclusion in each case was that the microenvironment had a pH below 5 (17–19).

There have also been efforts to use direct approaches to evaluate the pH within degrading polymer devices. In one study, microspheres incubated *in vitro* were dissolved in acetonitrile:water and the acidity of the organic phase was measured. Measurements were then correlated with the actual proton activity, and the microclimate pH was estimated to be approximately 1.8 (18). Encapsulation of fluorescein within the microsphere followed by confocal fluorescence microscopic imaging of the fluorescein emission intensity confirmed an acidic interior (18). In another study, NMR was employed to examine PLGA microspheres incubated in sheep serum. Results showed an average pH of 6.4 within the population of the microspheres over the course of 45 days (7). EPR has also been used to examine PLGA tablets and microspheres. The tablets implanted subcutaneously in mice indicated an average pH of 2 to 4 after 6 days (9). Using the same technique to probe *in vitro* degradation of microspheres in phosphate buffered saline (PBS), pH 7.4, the pH dropped to a value equal or below 4.7 over the course of the experiment (20,21), and an average pH of 3.5 was observed in microparticles containing spin-labeled albumin (22).

The results of these studies differ from each other most likely because the systems examined were different—relatively porous microspheres vs. dense microspheres and tablets—as were the incubation conditions—*in vitro* vs. *in vivo*. However, in all these studies, the experimental techniques could not quantitatively determine the spatial distribution of pH within a microsphere or throughout a device.

Although knowledge of an average pH of the system is useful, it is important to quantify the extremes of pH. A high average pH does not preclude the existence of pockets of high acidity within the device. The range of pH environments and the amount of time in that environment which a protein can tolerate are different for every protein. Thus, to approach the problem of protein stability within a polymer system it is necessary to quantify the lowest pH environment within the device

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as well as the length of time that this environment exists. Herein, we observe for the first time the quantitative, spatial distribution of pH changes within a population of polymeric microspheres over the course of their degradation.

MATERIALS AND METHODS

Materials

PLGA 50:50 (RG503, lot 34033) was purchased from Boehringer Ingelheim (Montvale, NJ) and had a number-average molecular weight of 25,000 Da. Poly(vinyl alcohol) (PVA) (88% hydrolyzed, 25,000 Da) was obtained from Polysciences (Warrington, PA). Carboxy SNARF-1 conjugated to 10,000 Da dextran (SNARF-dextran) and C1-NERF conjugated to 10,000 Da dextran (NERF-dextran) were from Molecular Probes (Eugene, OR). All other chemicals were reagent grade.

pH Electrode Measurements

pH electrode measurements were made essentially as described previously (17). Acetonitrile was added to a sample of 20 mg microspheres in 2 mL PBS (1 mM KH_2PO_4, 10 mM Na_2HPO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.4) to a final ratio of 4:1 (v/v). This solution was mixed for 15 sec, and the pH was measured using a glass electrode pH meter. To correlate this with a concentration of hydronium ions, standards were made up with known concentrations of lactic acid in a solution of acetonitrile:water, 4:1 (v/v). pH measurements of the standards were taken 15 sec after addition of acetonitrile. The pH meter measurements were then corrected to obtain the actual proton activity in the organic solution mixture. Samples were run in triplicate.

Preparation of Microspheres

Microspheres were prepared by a double-emulsion technique (23). To 100 μL of aqueous dye solution was added 1 mL of a solution of 200 mg/mL PLGA in methylene chloride, and the mixture was sonicated for six 0.5-second, 40-watt pulses with a Vibracell (Danbury, CT) sonicator using a microtip probe. This primary emulsion was then added to 20 mL of an aqueous solution of 2.5% (w/v) PVA. The second emulsion was formed by homogenization for 1 min at 5000 rpm on a Silverson (East Longmeadow, MA) homogenizer using a 5/8″ micro-mixing assembly with a general-purpose disintegrating head. The resultant water-in-oil-in-water (w/o/w) emulsion was then poured into 80 mL of 1% PVA and stirred continuously for 3 h to evaporate the solvent. The hardened microspheres were centrifuged, washed 3 times with water, freeze-dried, and stored under desiccant at −20°C.

Loading of Microspheres

Ten mg of dried microspheres were dissolved in 3 mL of 0.1 N NaOH/0.5% SDS overnight at 37°C. Samples were then diluted 1:9:9 with 1 mM HCl and PBS (at 10-fold concentration) so that all the samples were analyzed at the same pH to avoid artifacts due to the pH sensitivity of the dyes. The pH of the resulting solutions was measured using a standard glass electrode to ensure that the samples were all buffered to the same pH as the standards (pH 7.0). Standards were made by dissolving known amounts of dye in similar solutions. All samples were then measured for fluorescence intensity using a Photon Technology International fluorimeter (Ashland, MA) using λ_{ex} = 488 nm and λ_{em} of 580 nm (SNARF-dextran) and 535 nm (NERF-dextran). Measurements were related to the standard curve to determine dye content.

Release from Microspheres

Ten mg of dried microspheres were suspended in a round-bottom, 1.8-mL cryovial containing 1 mL of PBS, pH 7.4. Sample tubes were incubated at 37°C with continuous shaking. To sample the released dye, tubes were centrifuged and the supernatant was removed and analyzed. pH of the supernatant was measured to verify that it was at 7.4. The sample tubes were replenished with PBS, vortexed to resuspend the microspheres, and placed back in the incubator. Samples were run in triplicate.

Confocal Microscopy

Ratio imaging was conducted on a Bio-Rad (Hercules, CA) MRC 600 laser-scanning confocal microscope. The pH was determined using two different dyes; a combination of SNARF-dextran (λ_{em} = 580 nm) and NERF-dextran (λ_{em} = 535 nm) was used at a ratio of 2:1 (w/w). Images at the two wavelengths were collected in photon-counting mode using a 63x objective. The gain was set on 8.0 and the black level was set on 5.5 for each channel. Images in the two channels were then collected by accumulating to peak. The ratio was calculated using NIH Image 1.61 (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/). Calibration curves were generated for each dye set by placing solutions of dye at known pH on a microscope slide and focusing the microscope within the solution. Multiple images were taken for each pH value and then averaged to obtain the calibration curve.

Gel Permeation Chromatography

Microspheres pre-incubated in PBS were washed with water to remove buffer salts, freeze-dried, resuspended in chloroform and filtered. Samples were analyzed on two PL-Gel mixed-D columns in series (5 μm, 300 × 7.5 mm, Polymer Laboratories, Amherst, MA) and eluted with chloroform at 1 mL/min on a Perkin Elmer LC-250 with refractive index detection. Molecular weight was determined relative to polystyrene standards. All samples were run in triplicate.

Scanning Electron Microscopy (SEM)

Scanning electron micrographs were obtained using a JEOL JSM-6320 FV microscope at 0.8 kV. Microspheres were mounted in the powder form and were not coated.

Dye Binding to PLGA Microspheres

PVA-free microspheres made using a spray/freeze-drying technique (24) were used so that the dyes contacted polymer rather than PVA on the surface of the microspheres. SNARF-dextran/NERF-dextran solutions (2:1 w/w) were made in phosphate buffers (50 mM phosphate, 100 mM NaCl) at pH 2, 4

and 7.4 at concentrations of 0.5 and 0.05 μg/mL. The rationale for using 0.05 μg of dye with 10 mg of microspheres was to mimic similar loading conditions of dye within the microspheres. One mL of solution was added to 10 mg of microspheres and incubated at 37°C for 24 h with agitation. Samples were then centrifuged and the supernatant was measured for fluorescence intensity and compared with controls where dye was incubated without microspheres. Samples were run in triplicate.

RESULTS AND DISCUSSION

The technique of imaging pH-sensitive, fluorescent dyes conjugated to dextrans within microspheres using confocal microscopy was selected herein for several reasons: 1) it would allow determination of pH values throughout a microsphere as opposed to an average pH value within a population, 2) pH distributions could be determined at different planar sections within the microsphere, 3) ratiometric imaging provides quantitative information that is independent of dye concentration, and 4) the large dextran molecules conjugated to the dyes would prevent diffusion of the latter out of the microsphere during the course of the experiment.

Microspheres were prepared by double-emulsion, the most commonly used for protein encapsulation, and were incubated in aqueous solution under physiologic conditions, whereupon the microsphere becomes wetted and the PLGA undergoes hydrolysis. As the polymer chains of the microsphere degrade, either by end-chain or random-chain scission, smaller polymer chains are created. This increases the number of carboxylic end-groups and thus the total hydronium ion concentration within the particle.

Verification of pH Change Within the Microsphere

Prior to the microscopy measurements, we verified there was a pH change occurring within the microspheres. The bulk acidity of the microenvironment was determined by solubilizing the microspheres in aqueous acetonitrile and measuring pH with a glass electrode (see Methods). The results were compared with those of the buffered solution in which the microspheres were incubated. As seen in Fig. 1, there is a large difference between the buffered solution pH and that of solubilized microspheres after two weeks: the average pH within the microspheres dropped to 5.2, whereas that of the buffered solution remained at about 6.9. While the acidity of the entire system rose, the rate of increase within the microspheres was approximately double that of the external buffer, likely due to a sequestration of acidic monomers and oligomers in the polymer.

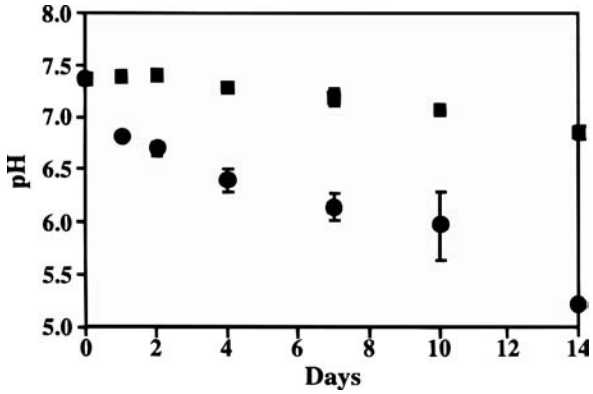

Fig. 1. pH measurements of solubilized microspheres in 4:1 acetonitrile:PBS (●) corrected against lactic acid standards measured in 4:1 acetonitrile:water and of PBS buffer in which the microspheres are incubated (■). Error bars represent standard error of 3–5 experiments.

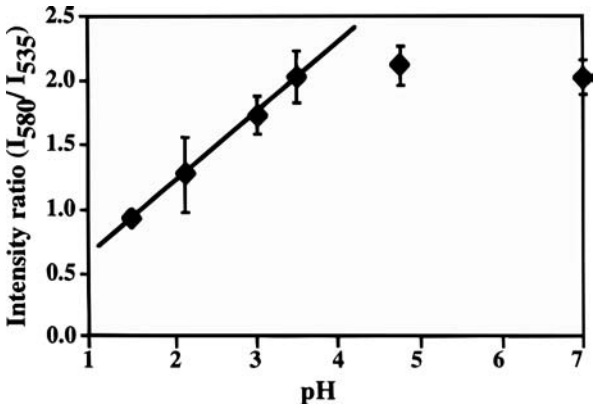

Fig. 2. Standard curve of fluorescence intensity ratios of SNARF-dextran/NERF-dextran (2:1 w/w) vs. pH. The equation for the fitted line is $y = 0.54x + 0.13$ with a r^2 value of 0.997. Results are the average of at least 3 images. Error bars represent standard error.

Development of Experimental Methods

Having confirmed the existence of an acidified environment within the particles, we next established suitable conditions, such as dye concentration and SNARF-dextran:NERF-dextran ratio, for the confocal microscopy experiments. We generated a standard curve wherein a combination of the SNARF-dextran and NERF-dextran dyes (2:1, w/w) was dissolved in phosphate buffers (50 mM phosphate/100 mM NaCl) with pH values, adjusted by addition of HCl, ranging from 1.5 to 7.0 and imaged with confocal microscopy. As shown in Fig. 2, the SNARF-dextran/NERF-dextran emission ratio linearly correlates with pH in the range from 1.5 to 3.5. (Standard curves were generated with every experiment.)

Several controls were performed to avoid artifacts. PLGA microspheres without dye (both wetted and dry) were imaged and found to have no fluorescence (Fig. 3a). Dry microspheres containing the dyes were imaged and again found to have no fluorescence (Fig. 3b). Dye loading within the microspheres was determined (0.05% w/w), and a 15-day release experiment

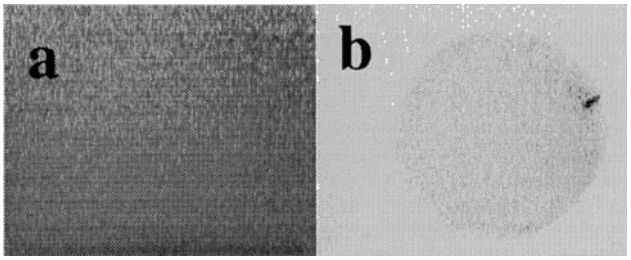

Fig. 3. (a) Ratio image of blank PLGA microsphere (containing no dye) which had not been incubated in PBS. (b) Ratio image of dye-containing microsphere which, again, had not been wetted with PBS.

confirmed that the dye did not leach out of the microspheres over the course of the experiment: less than 10% of the dye was released from the microspheres (data not shown).

There was a concern that the dyes could bind to the polymers and undergo a change in pK, thus altering their response to pH changes. To address this issue, the following experiments were conducted. The dyes were incubated with PLGA microspheres made in the absence of PVA (to test for binding of the dyes directly to PLGA and not to the PVA which is used as an emulsion stabilizer). The dyes were prepared at two different concentrations and in phosphate buffers at three different pHs, and incubated with the microspheres for 24 hours at 37°C. Dye concentrations in solution were then determined by fluorescence spectroscopy. The difference in fluorescence intensity between the control (dye incubated at identical conditions in the absence of spheres) and the supernatant of the experiment was found to be less than 6% (i.e., within the error of the measurements). This gave a strong indication that no appreciable interaction occurred between the dye and the polymer to affect the pH values. pH changes due to potential dielectric constant changes in the environment surrounding the probe could not be eliminated.

Kinetics of pH Change Within Microspheres

Using our technique, we monitored the change in pH within microspheres over time. Microspheres were incubated continuously for 15 days and imaged several times over that period. A distribution of fluorescence intensity ratios (related to pH) could be seen within the microsphere particles, as exemplified in Fig. 4. Over the incubation period, the pH within the microspheres drops precipitously, down to as low as 1.5 in the center of the microsphere (Fig. 5a–d). This data is in agreement with the previously published work (discussed earlier) where an acidic pH was detected within microsphere populations. The pH increases from the center of the bead to the edges due to diffusion of degradation products out of, and outside buffer components into, the microsphere, thus resulting in a concentration gradient of acidic by-products. Thus, a zone of higher pH exists near the surface which moves toward the center as the microsphere becomes more permeable.

The pH changes within the microspheres are dependent on the diameter of the polymer bead as well (Fig. 5e–h). In smaller spheres, clearance of acidic by-products is faster due to shorter diffusion distances, and thus only a small proportion of the bead is at a low pH.

To ensure that the inner-particle pH was not a reflection of the outer buffer pH, the latter was measured using a standard pH probe. After 15 days of incubation, the pH of the outer medium was well above that observed within the interior of the spheres (data not shown).

It was hypothesized that by daily changing the outer buffer in which the microspheres were incubated, with fresh PBS, the development of the low pH region would be retarded. Figures 5i–l indeed show a slower development of a low-pH region within the larger microspheres. However, by the end of the 15-day incubation, the results were the same as those in which the buffer was not changed daily. A highly acidic core had developed within the microspheres. Clearly, in the larger spheres the diffusion distance was long enough to prevent the buffer from neutralizing the pH. However, with smaller spheres, this was not the case. There was only a small volume with an acidic intrapolymer environment (Fig. 5m–o). Thus, one tactic to avoid an acidic microenvironment would be to use smaller spheres. In our case, for example, the spheres with a diameter of approximately 15 μm do not develop a significant volume of the low-pH region.

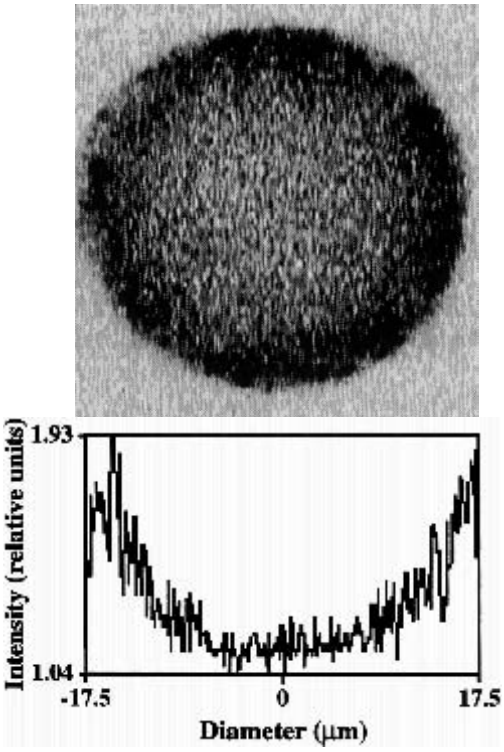

Fig. 4. (a) Ratio image of dye-containing PLGA microsphere incubated in PBS for 8 days. (b) Fluorescence intensity profile across the diameter of the microsphere quantified using NIH Image.

Our findings are summarized in Fig. 6, which depicts three spheres incubated for 10 days. This figure shows clearly the difference in pH distributions due to the size of the microspheres. As the size of the microsphere decreases, the fraction of sphere volume at neutral pH increases. As an approximation, an average pH was calculated for each particle by assuming that the dark regions were of a pH of 7.4 and integrating the pH of the light region using NIH Image. For the largest bead, the calculated average pH was 5.4, for the medium-sized particle it was 6.2, and the smallest particle had an average pH of 7.2. The pH of 6.2, determined for the medium-sized sphere, is similar to the average pH value found in other PLGA microsphere systems, although it is important to emphasize that the microspheres of that study (7) were manufactured by a different method (different processing methods give rise to microspheres with distinct structural properties, such as density or porosity). However, it is evident from these results that a large portion of a microsphere can contain an acidic region but still maintain a high average pH.

Porosity of the microsphere structure would likely play a significant role in determining the acidity of the intraparticle environment. In more porous structures, monomers and oligomers would have a larger flux out and buffer salts could more easily diffuse in, thus mitigating acidification of the polymer

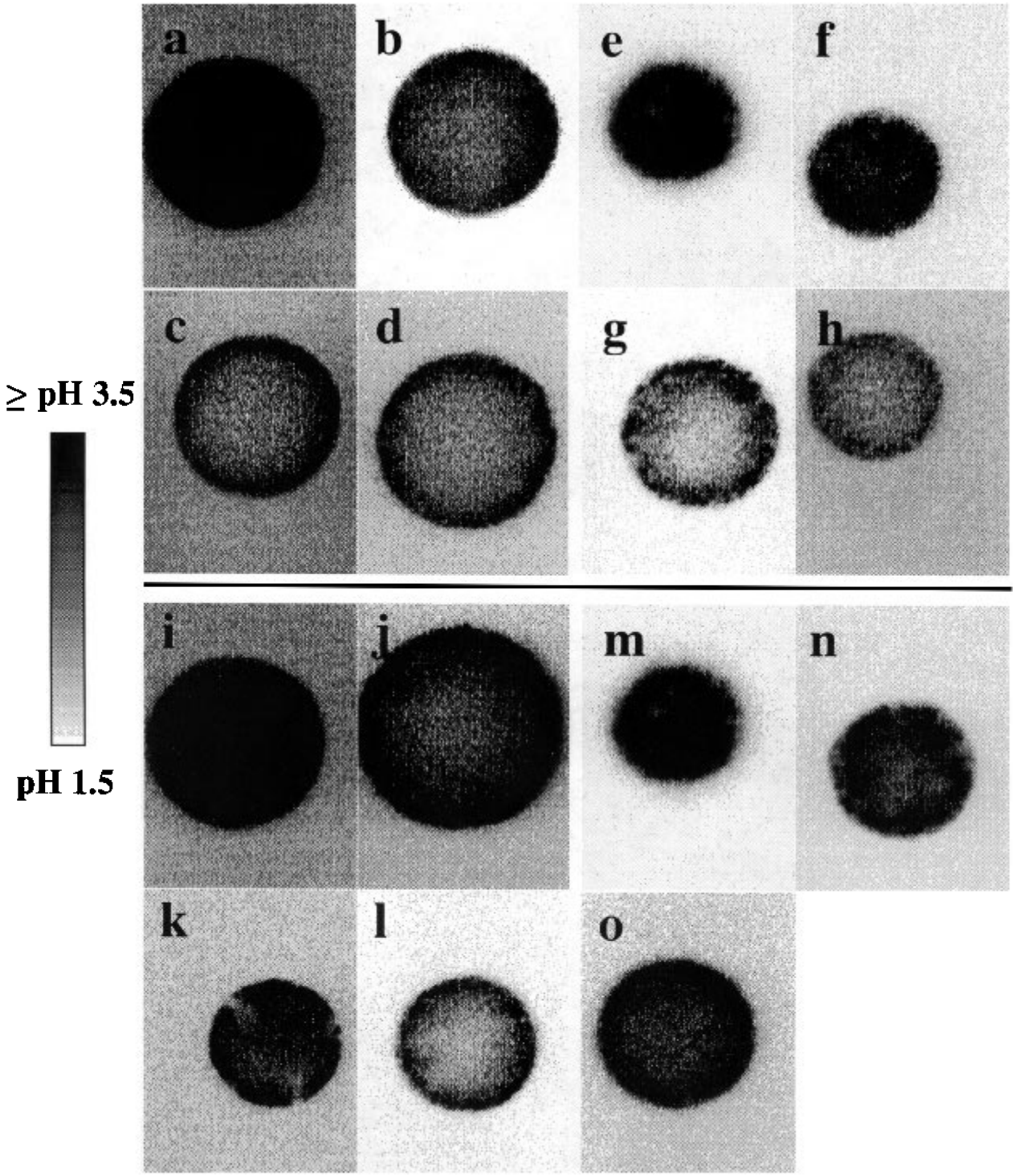

Fig. 5. Large and small microspheres incubated either continuously for 15 days in PBS which either was not changed (a–h) or was changed daily (i–o). Images a–h were taken on day (a) 1 (d = 40 μm), (b) 2 (d = 40 μm), (c) 10 (d = 40 μm), (d) 15 (d = 46 μm), (e) 1 (d = 29 μm), (f) 2 (d = 25 μm), (g) 10 (d = 32 μm), and (h) 15 (d = 31 μm). Images i–o were taken on day (i) 1 (d = 40 μm), (j) 3 (d = 46 μm), (k) 10 (d = 40 μm), (l) 15 (d = 40 μm), (m) 1 (d = 29 μm), (n) 2 (d = 25 μm), and (o) 10 (d = 30 μm). Standard curve is represented by a grayscale curve as shown on the left.

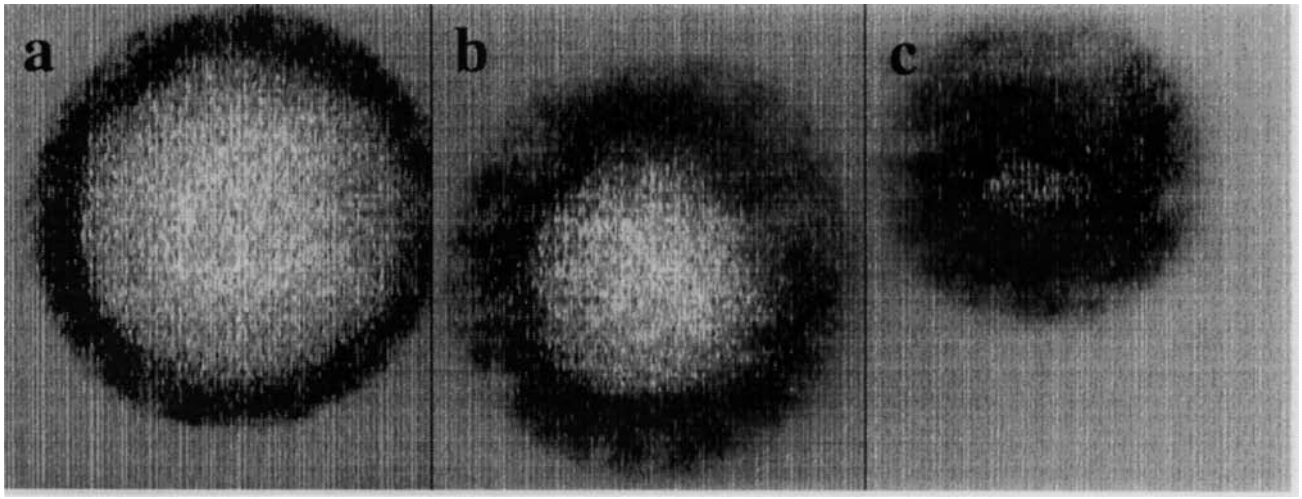

Fig. 6. Three microspheres of different sizes imaged after 10 days of incubation in PBS without any change of buffer. (a) d = 38 μm, (b) d = 24 μm, (c) d = 14 μm.

interior. In contrast, more dense structures, like the microspheres shown here, retain their degradation products giving rise to microenvironments with high acidity.

The protein encapsulated will also contribute to the intraparticle environment since certain proteins can serve as buffers to neutralize the pH within the microsphere. We are currently conducting studies measuring the pH within microspheres that contain both the pH-sensitive fluorescent dyes as well as a protein drug.

Physical Properties of Degrading Microspheres

Another way to examine the issue of low-pH environments within PLGA particles is to directly characterize polymer degradation and loss of particle mass. We observed by SEM (Fig. 8) that the microsphere structure does not seem to be altered after incubation for 15 days. The core of the microsphere appears to remain dense with few visible pores. This implies that the polymer chains are long, on average, and that little polymer has been lost during degradation. By quantifying the molecular weight of the polymer chains of the microsphere and the dry weight of the microspheres over time, we directly examined these issues, as shown in Fig. 8. Over the 15-day degradation, the dry weight of the microspheres remained constant, while the number- and weight-average molecular weight of the polymer chains dropped precipitously. This corresponds well with the conclusions drawn from the scanning electron micrographs.

Extension of this technique to other polymers and formulations should increase our understanding of the environment within the devices, as well as the process of polymer degradation. Models of the degradation, including accumulation of acidic by-products, could be developed to provide insight into the kinetics of polymer erosion and the patterns of drug release.

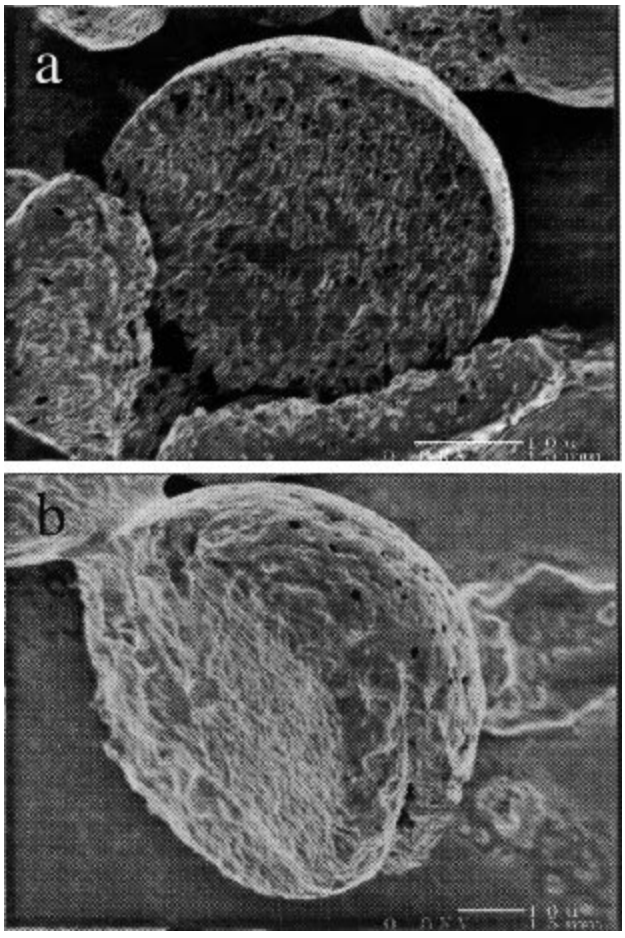

Fig. 7. Scanning electron micrographs of fractured microspheres. Samples were mounted dry and were not coated. (a) Microsphere which had not been incubated. (b) Microsphere which had been incubated for 15 days in PBS.

CONCLUSIONS

By encapsulating pH-sensitive, fluorescent dyes within PLGA microspheres and imaging them with confocal microscopy, we have established a method to quantitatively determine

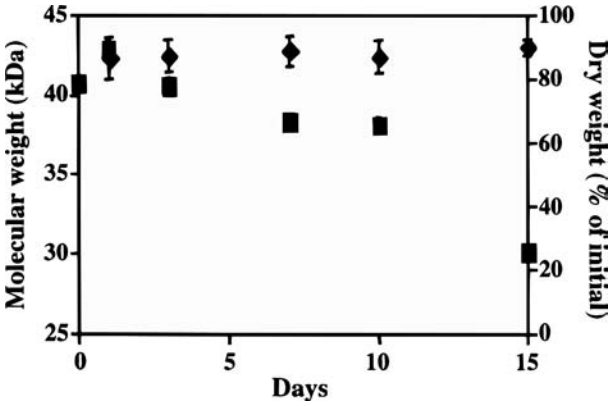

Fig. 8. Degradation of PLGA microspheres over 15 days in PBS. (■) Dry weight of microspheres over the course of incubation. (♦) Molecular weight of polymer chains of microspheres as determined by GPC. Error bars represent standard error of three experiments.

the pH (in the range of 1.5–3.5) within a microsphere particle. This is a general method that can be applied to different polymer systems as well as device morphologies. After incubation for several days under physiologic conditions, an acidic region developed within the center of the microspheres. The fraction of sphere volume comprising the acidic region decreased with decreasing bead diameter. The pH within the larger (~40 μm) particles was determined to be as low as 1.5, which is sufficiently acidic to cause denaturation of, and/or deleterious reactions in, many potential protein therapeutics. The area of low pH diminished in size and eventually vanished with prolonged incubation (>15 days) of the particles. These results are consistent with a diffusion-controlled mechanism in which acidic degradation products initially diffuse out of the bead slowly. As degradation proceeds, the polymer matrix becomes less dense, allowing increased diffusion and ultimately free exchange of monomers and buffer salts.

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REFERENCES

1. K. Fu, D. Pack, A. Laverdiere, S. Son, and R. Langer. Visualization of pH in degrading polymer microspheres. *Proceed. Int'l. Symp. Control. Rel. Bioact. Mater.* **25**:150–151 (1998).
2. R. Langer. Controlled release of a therapeutic protein. *Nature Medicine.* **2**:742–743 (1996).
3. N. Peppas and R. Langer. New Challenges in Biomaterials. *Science.* **263**:1715–1720 (1994).
4. R. Langer and J. Vacanti. Tissue engineering. *Science.* **260**:920–926 (1993).
5. E. Mathiowitz, J. Jacob, Y. Jong, G. Carino, D. Chickering, P. Chaturvedi, C. Santos, K. Vijayaraghavan, S. Montgomery, M. Bassett, and C. Morrell. Biologically erodable microspheres as potential oral drug delivery systems. *Nature.* **386**:410–414 (1997).
6. I. Grizzi, H. Garreau, S. Li, and M. Vert. Hydrolytic degradation of devices based on poly(DL-lactic acid) size-dependence. *Biomaterials.* **16**:305–311 (1995).
7. P.A. Burke. Determination of internal pH in PLGA microspheres using 31P NMR spectroscopy. *Intern. Symp. Control. Rel. Bioact. Mater.* **23**:133–134 (1996).
8. M. Vert, S. Li, and H. Garreau. More about the degradation of LA/GA-derived matrices in aqueous media. *J. Contr. Rel.* **16**:15–26 (1991).
9. K. Mader, B. Gallez, K. J. Liu, and H. M. Swartz. Non-invasive *in vivo* characterization of release processes in biodegradable polymers by low-frequency electron paramagnetic resonance spectroscopy. *Biomaterials.* **17**:457–461 (1996).
10. A. Domb, L. Turovsky, and R. Nudelman. Chemical interactions between drugs containing reactive amines with hydrolyzable insoluble biopolymers in aqueous solutions. *Pharm. Res.* **11**:865–868 (1994).
11. J. Cleland, M. Powell, and S. Shire. The development of stable protein formulations: a close look at protein aggregation, deamidation, and oxidation. *Crit. Rev. Therapeutic Drug Carrier Systems.* **10**:307–377 (1993).
12. C. Middaugh, R. Evans, D. Montomgery, and D. Casimiro. Analysis of plasmid DNA from a pharmaceutical perspective. *J. Pharm. Sci.* **87**:130–146 (1998).
13. G. Zhu and S. P. Schwendeman. Influence of basic salts on stability and release of proteins in injectable poly(lactide-co-glycolide) delivery devices. *Proceed. Int'l. Symp. Control. Rel. Bioact. Mater.* **26**:1114–1115 (1999).
14. G. Zhu and S.P. Schwendeman. Stabilization of bovine serum albumin encapsulated in injectable poly(lactide-co-glycolide) millicylinders. *Proceed. Int'l. Symp. Rel. Bioact. Mater.* **25**:267–268 (1998).
15. A. Goepferich. Mechanisms of polymer degradation and erosion. *Biomaterials.* **17**:103–114 (1996).
16. T. Uchida, A. Yagi, Y. Oda, Y. Nakada, and S. Goto. Instability of bovine insulin in poly(lactide-co-glycolide) (PLGA) microspheres. *Chem. Pharm. Bull.* **44**:235–236 (1996).
17. A. Shenderova, T. G. Burke, and S. P. Schwendeman. Evidence for an acidic microclimate in PLGA microspheres. *Proceed. Int'l. Symp. Control. Rel. Bioact. Mater.* **25**:265–266 (1998).
18. A. Shenderova, T. G. Burke, and S. P. Schwendeman. The acidic microclimate in poly(lactide-co-glycolide) microspheres stabilizes camptothecins. *Pharm. Res.* **16**:241–248 (1999).
19. A. Shenderova, M. Madou, S. Yao, and S. P. Schwendeman. Potentiometric and impedance measurements of PLGA coated microelectrodes. *Proceed. Int'l. Symp. Control Rel. Bioact. Mater.* **26**:727–728 (1999).
20. A. Brunner, K. Maeder, and A. Goepferich. The chemical microenvironment inside biodegradable microspheres during erosion. *Proceed. Int'l. Symp. Control. Rel. Bioact. Mater.* **25**:154–155 (1998).
21. A. Brunner, K. Maeder, and A. Goepferich. pH and osmotic pressure inside biodegradable microspheres during erosion. *Pharm. Res.* **16**:847–853 (1999).
22. K. Maeder, B. Bittner, Y. Li, W. Wohlauf, and T. Kissel. Monitoring microviscosity and microacidity of the albumin microenvironment inside degrading microparticles from poly(lactide-co-glycolide) (PLG) or ABA-triblock polymers containing hydrophobic poly(lactide-co-glycolide) A blocks and hydrophilic poly(ethyleneoxide) B blocks. *Pharm. Res.* **15**:787–793 (1998).
23. S. Cohen, T. Yoshioka, and R. Langer. Controlled delivery systems for proteins based on poly(lactic/glycolic acid) microspheres. *Pharm. Res.* **8**:713–720 (1991).
24. M. Tracy. Development and scale-up of a microsphere protein delivery system. *Biotechnol. Prog.* **14**:108–115 (1998).